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The Nuclear Receptor-Coactivator Interaction Surface as a Target for Peptide Antagonists of the Peroxisome Proliferator-Activated Receptors

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The peroxisome proliferator-activated receptors (PPAR α , PPAR δ , and PPAR γ) constitute a family of nuclear receptors that regulates metabolic processes involved in lipid and glucose homeostasis. Although generally considered to function as ligand-regulated receptors, all three PPARs exhibit a high level of constitutive activity that may result from their stimulation by intracellularly produced endogenous ligands. Consequently, complete inhibition of PPAR signaling requires the development of inverse agonists. However, the currently available small molecule antagonists for the PPARs function only as partial agonists, or their efficacy is not sufficient to inhibit the constitutive activity of these receptors. Due to the lack of efficacious antagonists that interact with the ligand-binding domain of the PPARs, we decided to target an interaction that is central to nuclear receptor-mediated gene transcription: the nuclear receptor-coactivator

interaction. We utilized phage display technology to identify short LXXLL-containing peptides that bind to the PPARs. Analysis of these peptides revealed a consensus binding motif consisting of HPLLXXLL. Cross-screening of these peptides for binding to other nuclear receptors enabled the identification of a high-affinity PPAR-selective peptide that has the ability to repress PPAR γ 1-dependent transcription of transfected reporter genes. Most importantly, when introduced into HepG2 cells, the peptide inhibited the expression of endogenous PPAR γ 1 target genes, adipose differentiation-related protein and mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A synthase 2. This work lends support for the rational development of peptidomimetics that block receptor-mediated transcription by targeting the nuclear receptor-coactivator interaction surface. (*Molecular Endocrinology* 21: 2361–2377, 2007)

THE PEROXISOME PROLIFERATOR-activated receptors α , δ , and γ (PPAR α or NR1C1, PPAR δ or NR1C2, and PPAR γ or NR1C3) constitute a family of

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Abbreviations: aa, Amino acid; ADRP, adipose differentiation-related protein; AF-2, activation function-2; AR, androgen receptor; ASC-2, activating signal cointegrator 2; BAP, biotin acceptor peptide; 9cisRA, 9-cis retinoic acid; CMV, cytomegalovirus; DR1, direct repeat 1; ER, estrogen receptor; ERR, estrogen-related receptor; 5-FAM, 5-carboxyfluorescein; Gal4DBD, Gal4 DNA binding domain; HMGCS2, 3-hydroxy-3-methylglutaryl coenzyme A synthase 2; HRP, horseradish peroxidase; LBD, ligand-binding domain; LRH-1, liver receptor homolog-1; LXR, liver X receptor; NLA, normalized luciferase activity; PBST, PBS + 0.1% Tween 20; PEPCK, phosphoenolpyruvate carboxykinase; PGC, PPAR γ coactivator; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoic X receptor; Sf9 cells, *Spodoptera frugiperda* cells; SRC-1, steroid receptor coactivator 1; TR, thyroid receptor; TRAP, TR-associated protein.

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nuclear receptors that bind endogenous lipid ligands and regulate metabolic processes involved in lipid and glucose homeostasis (1, 2). PPAR α is expressed at high levels in metabolically active tissues, reflecting its role in regulating genes that control fatty acid catabolism (3, 4). PPAR δ is expressed ubiquitously and regulates a variety of processes, including wound healing, keratinocyte differentiation, and angiogenesis (5–8). Whereas PPAR γ 1 is expressed at low levels in many tissues and plays roles in proliferation and differentiation, PPAR γ 2 is highly adipose specific, where it functions as the master regulator of adipogenesis (9–11).

The PPARs heterodimerize with the retinoid X receptor (RXR) and activate transcription by binding to PPAR response elements (PPREs) that contain a direct repeat 1 (DR1) element in the promoters of target genes. Modulation of nuclear receptor transcriptional activity has traditionally been accomplished using agonists and antagonists that bind to the ligand-binding domain (LBD) of the receptor and induce conformational changes that either facilitate or inhibit transcription. The PPARs can bind a variety of fatty acid

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ligands, although it is not known which of these serve as true endogenous ligands (12–15). Synthetic agonists have also been developed for the PPARs: fibrates including clofibrate and urea-substituted thioisobutyric acids such as GW7647 are used to activate PPAR α , carbaprostacyclin is an agonist of PPAR δ , and thiazolidinediones including rosiglitazone and pioglitazone manifest their antidiabetic actions through activation of PPAR γ 1 and PPAR γ 2 (16, 17).

Because these receptors exhibit a high level of constitutive activity, inverse agonists are needed to repress both the ligand-activated and constitutive activities of these receptors. However, the available small molecule antagonists for the PPARs function only as partial agonists, or their efficacy is not sufficient to inhibit the constitutive activity. Thus, there is a well-defined need for small molecules that can be used to inhibit PPAR constitutive activity.

Due to difficulties in developing efficacious small molecule antagonists of the PPARs that target the LBD, we decided to approach the development of antagonists of these receptors by targeting the nuclear receptor-coactivator interaction. Binding of agonist to the PPARs facilitates a conformational change in the activation function-2 (AF-2) domain of the receptor, which promotes the displacement of corepressor proteins (18, 19). Agonist binding further enables the presentation of a hydrophobic coactivator binding pocket, which facilitates the interaction between the nuclear receptor and the canonical helical LXXLL motif found within many coactivators (20–22).

Using phage display technology, our laboratory has previously identified LXXLL-containing peptides that interact with the AF-2 of estrogen receptor α (ER α), estrogen receptor β (ER β), androgen receptor (AR), vitamin D receptor, liver receptor homolog-1 (LRH-1), and estrogen-related receptor α (ERR α) (23–30). These respective peptides repress nuclear receptor-mediated transcription of transfected reporter genes. However, to our knowledge, peptide antagonists of nuclear receptors have not yet been successfully employed to block nuclear receptor-mediated regulation of endogenous target genes, an activity that must be demonstrated to validate the general approach.

Thus, the objectives of the current study were to 1) identify PPAR AF-2-interacting peptides using combinatorial phage display, 2) use these peptides to probe the nuclear receptor-coactivator interface, and 3) determine whether these peptides possess high enough affinity and selectivity to enable their use as peptide antagonists that repress AF-2-dependent reporter and endogenous gene transcription. The results of this study will assist in the development of peptidomimetics as PPAR modulators. Furthermore, they highlight the untapped potential of targeting the nuclear receptor-coactivator interface as a mechanism by which to modulate nuclear receptor transcriptional activity.

RESULTS

Selection of High-Affinity PPAR γ 1 Peptides Using Combinatorial Phage Display

To identify high-affinity peptides that interact with PPAR γ 1, we used combinatorial phage display technology to screen phage libraries expressing peptides as fusions with the M13 phage coat pili proteins (23, 25, 26). This method has been used previously by our laboratory to identify peptides that bind to ER α , ER β , AR, vitamin D receptor, LRH-1, ERR α , and retinoic acid receptor α (RAR α) (23–31). It has been established that PPAR γ 1 binds to LXXLL-containing coactivators, including p160 coactivators, PPAR γ coactivator 1 α (PGC-1 α), and thyroid hormone receptor-associated protein complex 220 kDa component (TRAP220) (32–34). Therefore, we elected to identify PPAR γ 1-interacting peptides by screening a phage library expressing 19 amino acid peptides with a fixed LXXLL amino acid motif flanked by seven random amino acids on either side of the motif (X₇-LXXLL-X₇). Because RXR α ligands can activate PPAR γ target genes, we hypothesized that the conformation of the AF-2 of PPAR γ 1 might be affected by its heterodimeric partner (35, 36). Therefore, to increase the diversity of the peptides identified, we screened for peptides that bound either to the PPAR γ 1/RXR α heterodimer on the PPRE from the adipocyte gene aP2, or to PPAR γ 1 that was immobilized directly on plastic.

To accomplish these objectives, highly purified recombinant PPAR γ 1 and RXR α were required. We produced full-length biotinylated human PPAR γ 1 (hPPAR γ 1) and RXR α (hRXR α) proteins by baculoviral overexpression in *Spodoptera frugiperda* (Sf9) cells and purified these biotinylated proteins on either monomeric avidin resin or streptavidin mitein matrix, as previously described for the purification of full-length androgen receptor (37). The purity of the recombinant hPPAR γ 1 and hRXR α proteins was evaluated by Coomassie blue staining (Fig. 1, A and B). The 475-amino acid hPPAR γ 1 protein fused to the 30-amino acid biotin acceptor peptide (BAP) was eluted from the column as a dominant 56-kDa band in the first eight fractions (Fig. 1A). A single band of the 462-amino acid hRXR α protein fused to BAP was detected at 55 kDa in four purified fractions (Fig. 1B).

To determine whether the proteins were functional with respect to their ability to heterodimerize on DNA, the hPPAR γ 1/hRXR α heterodimer was tested for its ability to bind to an aP2 PPRE oligonucleotide (Fig. 1C). The biotinylated oligonucleotide was immobilized on neutravidin-coated wells, and after excess neutravidin sites were blocked with biotin, hPPAR γ 1 and hRXR α were allowed to heterodimerize on the aP2 PPRE. GW7845, a nonthiazolidinedione agonist of PPAR γ , and 9-cis retinoic acid (9cisRA), an agonist of RXR α , were used to induce active conformations in each receptor (38, 39). As expected, only the apo- and GW7845-bound hPPAR γ 1/hRXR α heterodimers

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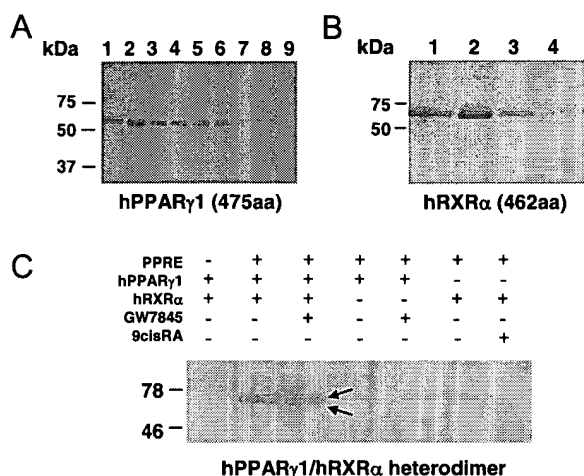


Fig. 1. Purification and Characterization of Purified hPPAR γ 1 and hRXR α

A and B, Full-length human hPPAR γ 1 and hRXR α proteins were overexpressed by infecting Sf9 insect cells with baculovirus expressing either hPPAR γ 1 or hRXR α . Proteins were purified by affinity chromatography using monomeric avidin resin and eluting with biotin-containing elution buffer. The elution fractions of hPPAR γ 1 (A, fractions 1–9) and of hRXR α (B, fractions 1–4) were loaded on reducing SDS-PAGE gels, and the purified proteins (fused to BAP) were visualized by Coomassie blue staining. C, The ability of hPPAR γ 1 and hRXR α to heterodimerize on an aP2 PPRE was tested *in vitro* in the absence or presence of PPAR γ agonist GW7845 or RXR α agonist 9cisRA, as indicated. The bound proteins were boiled in sample loading buffer, loaded on a reducing SDS-PAGE gel, and visualized by Coomassie blue staining. Arrows indicate the two bands of the tight doublet formed by hPPAR γ 1 and hRXR α (each fused to BAP).

(lanes 2 and 3) were capable of binding to the aP2 PPRE, as evidenced by the tight double band seen on a Coomassie blue-stained gel (Fig. 1C). This result is in agreement with the known ability of the PPAR γ 1/RXR α heterodimer to bind to DNA in either the absence or presence of ligand (40). Binding of the heterodimer was DNA dependent (lane 1), and hPPAR γ 1 and hRXR α were incapable of binding to the aP2 PPRE as monomers (lanes 4–7).

These data confirm the production of full-length hPPAR γ 1 and hRXR α proteins, demonstrate their functionality, and validate our screening conditions. We used these proteins to perform three rounds of panning of an M13 phage library to identify LXXLL-containing peptides that interact with either hPPAR γ 1 in the presence of vehicle or rosiglitazone, or with the hPPAR γ 1/hRXR α heterodimer in the presence of vehicle, rosiglitazone, or rosiglitazone plus 9cisRA. The enrichment of PPAR γ 1-binding phage was demonstrated by a phage ELISA (data not shown).

Peptides Interact with the PPARs in Cells

To determine whether individual peptides from the phage pools could interact with PPAR γ 1 in a cellular

environment, we used mammalian two-hybrid assays. DNA encoding peptides was PCR amplified from the pooled phage and shotgun ligated into a vector (pM5.1) that enabled the peptides to be expressed as fusion proteins at the N terminus of the Gal4 DNA binding domain (peptide-Gal4DBD). A total of 540 peptides were analyzed in mammalian two-hybrid assays performed in HepG2 cells for interaction with each of the PPARs fused to the VP16 transactivation domain. Mammalian two-hybrid analysis between five representative peptides (NBM131, NBM132, NBM225, NBM239, and NBM242) and VP16-PPARs showed robust interaction of each of these peptides with all of the PPAR subtypes (Fig. 2). The empty vector pM5.1 was used as a no-peptide control. No signal above background was detected when VP16 alone was used with any of these peptides, demonstrating that the interactions observed are PPAR dependent (data not shown).

Despite the fact that two of our five screens were performed in the absence of hRXR α protein, we found that 38% and 98% of all the peptides studied bound to VP16-RXR α in the presence of vehicle and 9cisRA, respectively (data not shown). This is not surprising because it is well known that LXXLL motifs bind to most nuclear receptors. To rule out the possibility, however, that these peptides may bind VP16-RXR α indirectly through binding to endogenous PPARs present in HepG2 cells, we repeated the analysis of several representative peptides with VP16-RXR α in HeLa cells, which do not express appreciable levels of the PPARs. The binding of the peptides to VP16-RXR α was reproduced in HeLa cells, suggesting that the binding to VP16-RXR α is, in fact, direct (data not shown).

Identification of an Extended PPAR Consensus Binding Motif

To determine the coactivator peptide-binding preferences of the PPARs, sequence analysis was performed on peptides that interacted with PPAR γ 1 in mammalian two-hybrid assays. Of the 130 peptides sequenced, 53 exhibited unique sequences (Fig. 3A). Alignment of these sequences revealed a strong consensus motif, HPLLXXLL (Fig. 3B). All of the sequences had a P in the –2 position with respect to the LXXLL motif, and only 13 had an amino acid other than H in the –3 position (M, N, S, or Y). More variability was observed for the –1 position with L being the most common amino acid, but other residues were observed as well (F, G, M, R, S, W, or Y) (Fig. 3, A and B). Interestingly, some flexibility was evident at the +4 and +5 positions, because peptides with AL, FL, ML, YL, and LI at these residues also interacted with PPAR γ 1 (Fig. 3, A and B).

To determine whether an unbiased, *i.e.* non-LXXLL-focused, screen would also select for peptides with the HPLLXXLL motif, we performed an additional screen of a peptide library with the format of SS-X₁₆-S

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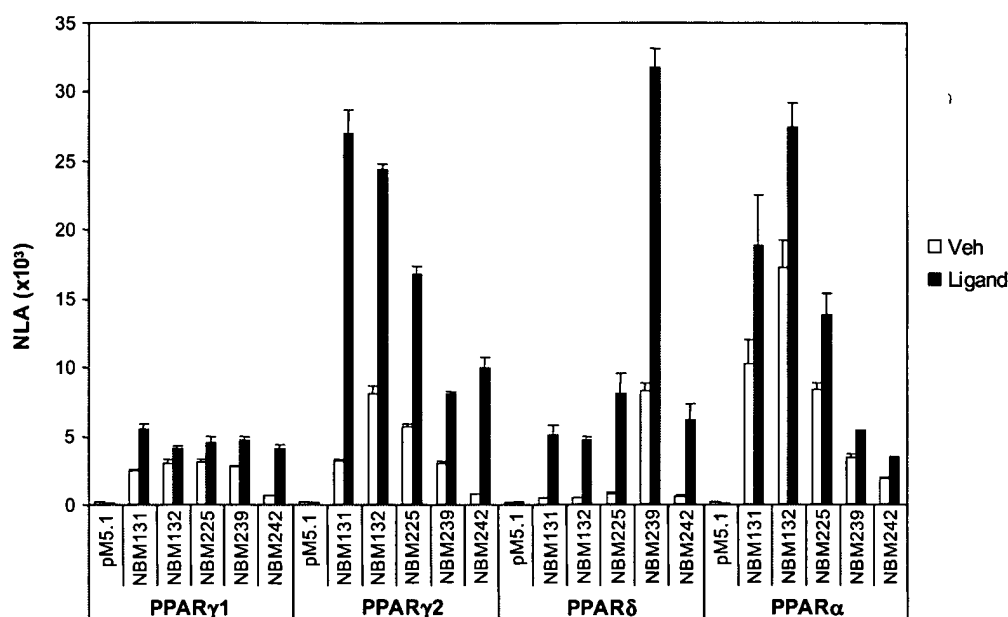


Fig. 2. Peptides Interact with the PPARs in Cells

Peptides ($n = 540$) were cloned as fusion proteins with the Gal4DBD. Mammalian two-hybrid analysis was performed to determine whether the peptide-Gal4DBD fusions could interact with the nuclear receptor fused to VP16 transactivation domain (VP16-NR) and activate transcription of a 5XGal4TataLuc3 reporter gene. Mammalian two-hybrid analysis of representative peptides was performed by transfecting HepG2 cells with VP16-GwA or VP16-GwA-NR (PPAR γ 1, PPAR γ 2, PPAR δ , PPAR α), 5XGal4TataLuc3, pM5.1 or pM5.1-peptide (NBM131, NBM132, NBM225, NBM239, NBM242), and CMV- β gal. White bars (\square) were treated with vehicle (Veh), black bars (\blacksquare) were treated with Ligand: 1 μ M rosiglitazone (PPAR γ 1 and PPAR γ 2), 10 μ M carbaprostacyclin (PPAR δ), or 100 μ M clofibrate (PPAR α). Peptide sequences are as follows: ATTPPTQLHPLLTLQFLRTD (NBM131), SSLEHPWLTMLLGKPHNMW (NBM132), PVFEHPRLVGLLVGTGDIAS (NBM225), ILEEHPLLASLLTETSRGM (NBM239), SLEAHPLLSALLQDQMGGD (NBM242). Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells.

(16 random residues flanked by SS at the N terminus and S at the C terminus). In addition, we also analyzed short peptides encoded by out-of-frame cDNAs recovered from another screen using several T7 cDNA libraries (Jansen, M. S., and D. P. McDonnell, unpublished data). Interestingly, both of these essentially random peptide screens also yielded peptides that contain sequences resembling the eight amino acid consensus (Fig. 3A).

The peptide libraries used in the screen consist of 19 amino acids; however, the consensus was defined by only eight amino acids (Fig. 3B). To test whether the eight-amino acid consensus motif is sufficient for binding to PPAR γ 1, the sequence corresponding to the motif derived from NBM242 was cloned as a fusion protein with the Gal4DBD. Mammalian two-hybrid analysis revealed that the eight-amino acid motif, HPLLALL, is sufficient for binding to PPAR γ 1 in a ligand-inducible manner (Fig. 3C).

We performed alanine scanning mutagenesis using the peptide NBM242 as a template to determine the contribution of each conserved residue in the consensus motif to PPAR γ 1 binding (Fig. 4A). Setting the first leucine of the LXXLL motif as position 1, the mutation number indicates the amino acid position at which the *in situ* amino acid was mutated to alanine (Fig. 4A). All

mutants were expressed as demonstrated by Western blot analysis with an anti-Gal4DBD antibody (Fig. 4B).

As expected, all three leucines within the LXXLL motif were indispensable for binding to the PPARs (Fig. 4C). Two control mutations upstream of the motif, mutation -5 and -6, retained binding to the PPARs. Mutational analysis revealed the importance of the -3 histidine, the -2 proline, and the -1 leucine for the binding of NBM242 to PPAR γ 1, PPAR α , and PPAR δ . Interestingly, mutation -6 increased the ligand-independent interaction of NBM242 with PPAR γ 1 and PPAR α to almost the level of interaction seen in the presence of their ligands.

The importance of these residues for binding to other nuclear receptors is more variable: mutation -3 permitted NBM242 to retain binding to RXR α , ER α , and ERR α , mutation -2 caused reduced binding, and mutation -1 caused reduced binding to RXR α and ER α but enabled NBM242 to retain some binding to ERR α (Fig. 4C). Unexpectedly, we found that the -6 leucine appears to be important for binding to ER α , because mutation of this amino acid drastically reduced binding of NBM242 to ER α . Collectively, these results demonstrate that although the -3 histidine, -2 proline, and -1 leucine are all important for NBM242 to bind to the PPARs, the -3 histidine is not required

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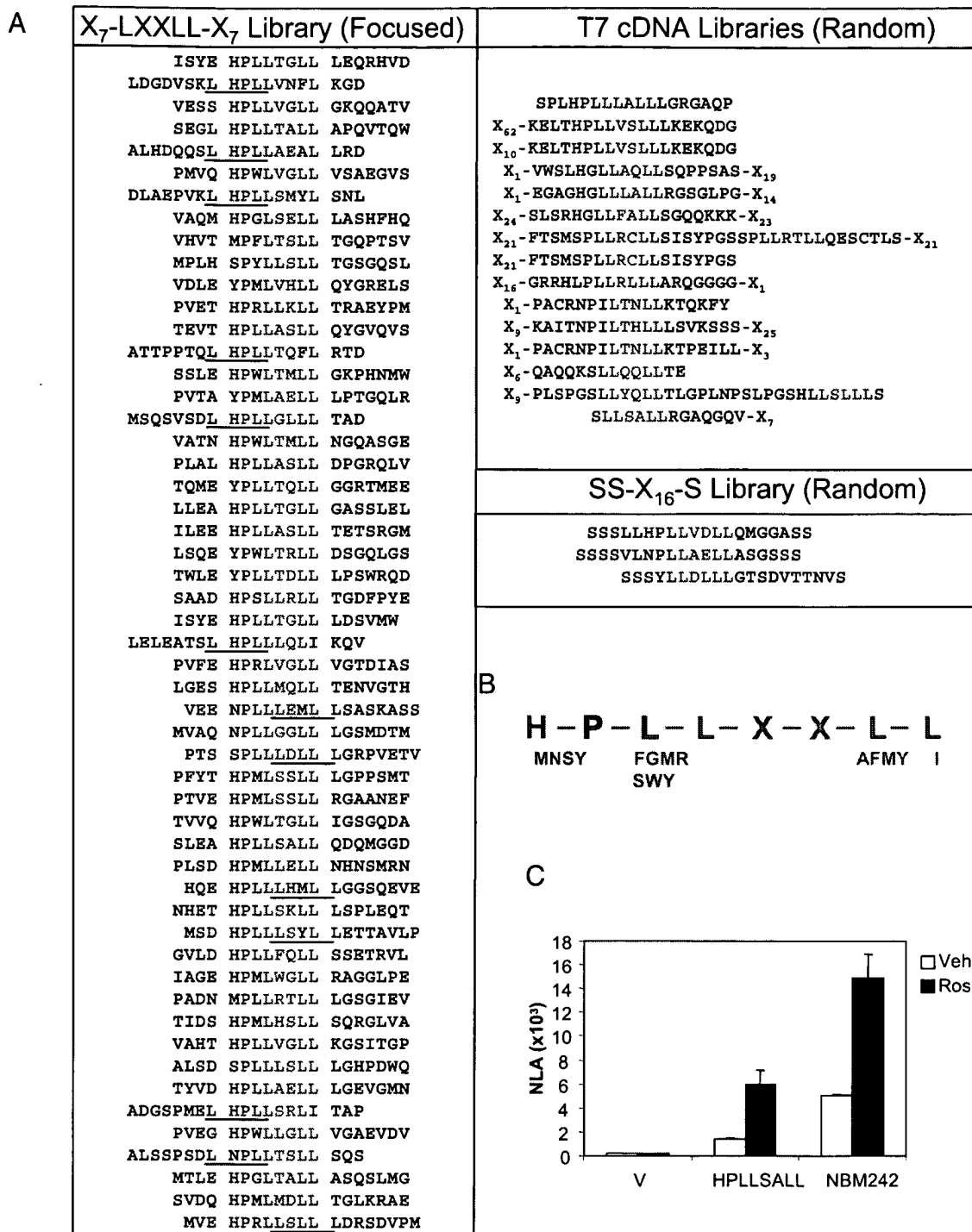


Fig. 3. Identification of an Extended PPAR Consensus Binding Motif

A, Sequence analysis of PPAR γ 1-interacting peptides isolated from two M13 peptide screens using X₇-LXXLL-X₇ and SS-X₁₆-S libraries, and from screens of T7 cDNA libraries. X_n denotes n number of amino acids. Peptides from the X₇-LXXLL-X₇ library have the fixed LXXLL motif shown in red, unless it is underlined in black. The extended part of the PPAR consensus binding motif is shown in blue. B, The sequences revealed an extended consensus binding motif consisting of eight amino acids. The other amino acids found occasionally in place of the -3 H, -1 L, +4 L, and +5 L are indicated below the motif. C, The eight-amino acid consensus motif from NBM242 was cloned as a fusion to the Gal4DBD, and the ligand-inducible interactions of vector (V), this eight-amino acid motif, HP LLSALL, as well as NBM242 with VP16-PPAR γ 1 were evaluated by mammalian two-hybrid analysis. HepG2 cells were transfected with VP16-PPAR γ 1, 5XGal4TataLuc3, pM5.1 (V) or pM5.1-peptide (HP LLSALL and NBM242), and CMV- β gal. White bars (□) were treated with vehicle (Veh), black bars (■) were treated with 1 μ M rosiglitazone (Rosi). Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells.

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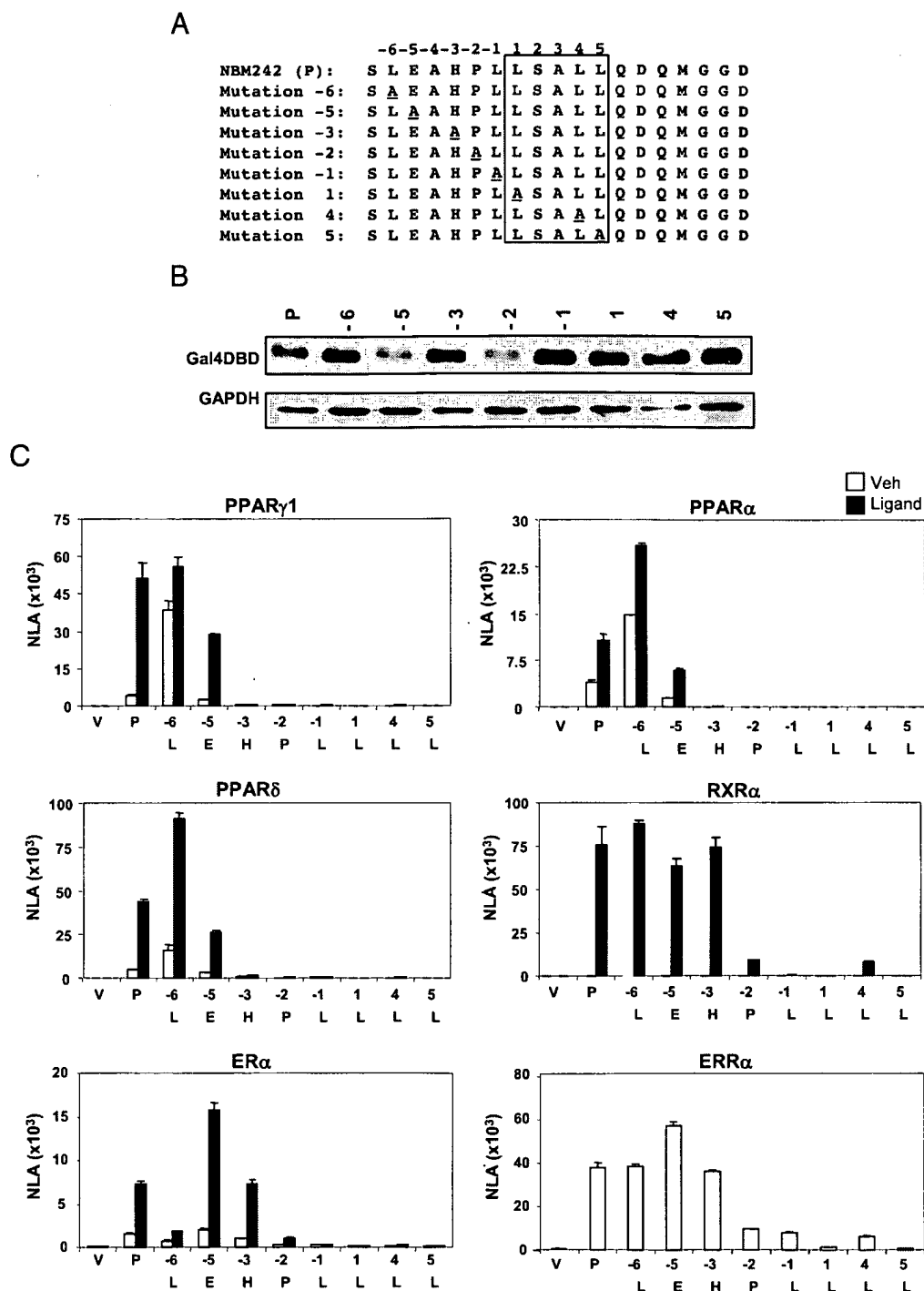


Fig. 4. The -3 Histidine Is Important for Binding to PPARs But Not to Other Nuclear Receptors

A, Alanine scanning was performed by site-directed mutagenesis of individual amino acids within the peptide NBM242 (P). The position of the amino acid mutations is indicated above the NBM242 sequence, using the first L of the LXXLL motif as position 1. The alanine mutation is *underlined* in each sequence, and the LXXLL motif is *boxed*. B, Expression of the mutants was confirmed by Western blot analysis using an anti-Gal4DBD primary antibody and an antirabbit IgG HRP-conjugated secondary antibody. GAPDH was used as a loading control. C, The mutants generated as depicted in panel A were evaluated by mammalian two-hybrid analysis for binding to VP16-PPAR γ 1, VP16-PPAR α , VP16-PPAR δ , VP16-RXR α , VP16-ER α , and VP16-ERR α . HepG2 cells were transfected with VP16-nuclear receptor, 5XGal4TataLuc3, pM5.1-peptide, and CMV- β gal. The mutation is indicated on the x-axis, where V is vector, P is the NBM242 peptide, and the number corresponds to the mutation number. The identity of the amino acid that was mutated is indicated *beneath* the mutation number. *White bars* (\square) were treated with vehicle (Veh), *black bars* (\blacksquare) were treated with ligand (1 μ M rosiglitazone for PPAR γ 1, 100 μ M clofibrate for PPAR α , 10 μ M carbaprostacyclin for PPAR δ , 1 μ M 9cisRA for RXR α , or 100 nM 17 β -estradiol for ER α). Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

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for the interaction of this peptide with other nuclear receptors including RXR α , ER α , and ERR α .

Stabilization of Peptides Occurs through Interaction of the –3 Histidine with K319 and E471 of PPAR γ 1

The importance of the –3 histidine was interesting, because this position has not been shown in previous studies to be important for interacting with other nuclear receptors (23–30). To understand how PPAR γ 1 may be interacting with this unique residue of the motif, we modeled the consensus peptide from NBM242 (HPLLSALL) with the PPAR γ LBD using the previously published structure of the PPAR γ /RXR α heterodimer complexed to 9cisRA, rosiglitazone, and a peptide derived from the p160 coactivator steroid

receptor coactivator 1 (SRC-1), containing a single LXXLL motif (41, 42). Based upon the modeling, the histidine within the motif is predicted to form a salt bridge with K319 within helix 4 and one of the charge clamp residues, E471, within helix 12 of PPAR γ 1 (Fig. 5A).

To determine the importance of K319 and E471 for stabilizing the interaction with PPAR-interacting peptides, each of these residues within PPAR γ 1 was mutated to alanine (K319A and E471A). Expression of the mutants was confirmed by Western blot analysis using an anti-PPAR γ antibody (Fig. 5B). Mammalian two-hybrid analysis was performed to test the ability of the mutated PPAR γ 1 receptors to bind to NBM242 (Fig. 5C, *left*). As expected, PPAR γ with the charge clamp glutamate mutation, E471A, completely lost the ability to interact with NBM242. K319A also destabilized but

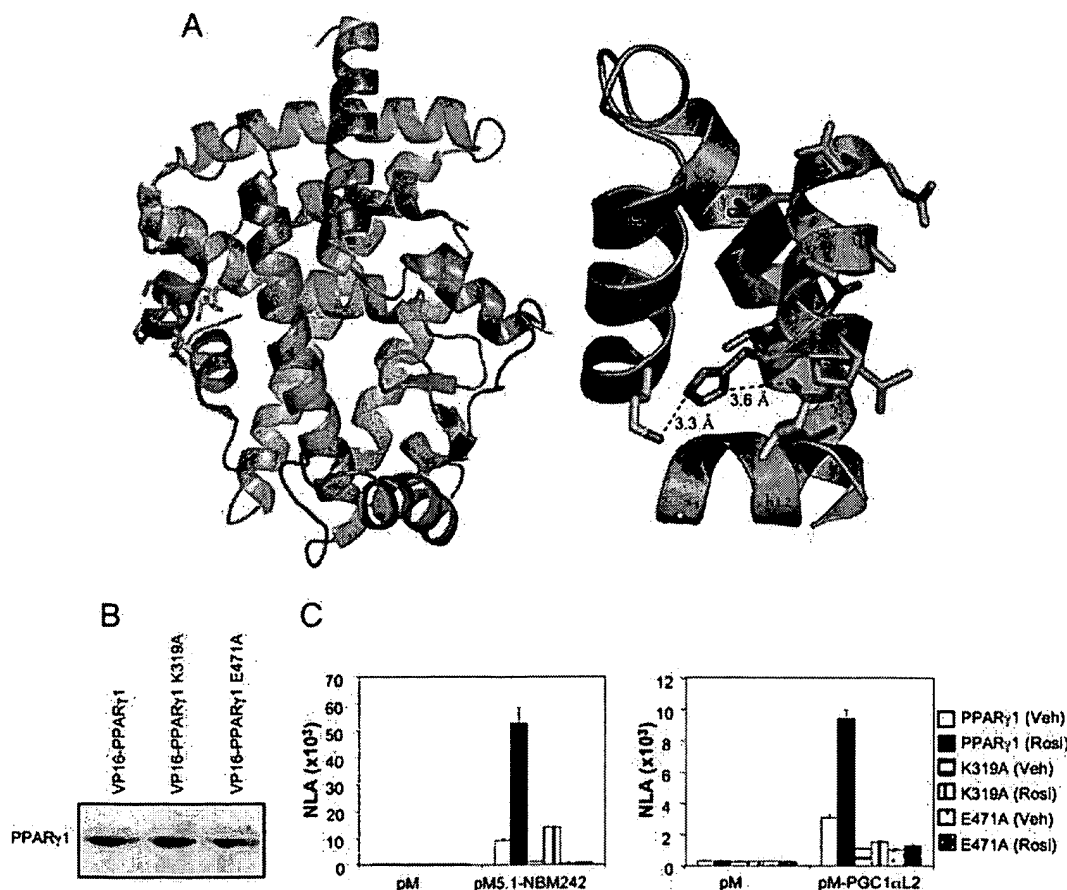


Fig. 5. Molecular Modeling of the NBM242 Peptide with PPAR γ LBD

A, The motif from the NBM242 peptide (HPLLSALL) was modeled using the published structure [PDB Code: 1FM6, Gampe et al. (42)] of the PPAR γ /RXR α heterodimer complexed to 9cisRA, rosiglitazone, and a peptide derived from the coactivator SRC-1, containing a single LXXLL motif (*left*) (41). Examined more closely (*right*), the histidine of the motif is predicted to form salt bridges with K319 in helix 4 and E471 in helix 12 of PPAR γ 1. B, VP16-PPAR γ 1 K319A and E471A mutants were made, and expression was confirmed by Western blot analysis with an anti-PPAR γ primary antibody and an antimouse IgG HRP-conjugated secondary IgG antibody. C, Interaction of the wild-type PPAR γ 1 (PPAR γ 1), PPAR γ 1K319A (K319A), and PPAR γ 1E471A (E471A) with NBM242 (*left*) and PGC-1 α L2 peptide (PGC1 α L2, EAEEPSLLKKLLAPANTQ, *right*) was tested by mammalian two-hybrid analysis in the presence of vehicle (Veh) or 1 μ M rosiglitazone (Ros). HepG2 cells were transfected with VP16 or VP16-PPAR γ 1 (PPAR γ 1, K319A, or E471A), 5XGal4TataLuc3, peptide (pM, pM-PGC1 α L2, or pM5.1-NBM242), and CMV- β gal. Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells.

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did not abolish the binding of NBM242 to PPAR γ 1, suggesting that there may also be other residues that are important for further stabilizing the interaction of PPAR γ 1 with the peptide.

Mammalian two-hybrid analysis with the 19-amino acid peptide centered around the second LXXLL motif of PGC-1 α (PGC1 α L2) also demonstrated that both the K319A and E471A mutations of PPAR γ 1 abrogated the interaction of the PGC1 α L2 peptide with PPAR γ 1. These data suggested that the peptides we identified bind to PPAR γ 1 in a manner similar to known PPAR γ 1 coactivators such as PGC-1 α (Fig. 5C, *right*).

PPAR Peptides Compete with Coactivators to Inhibit PPARE-Containing Reporter Gene Expression

The suggestion that our peptides might bind to PPAR γ 1 in a manner similar to known coactivators suggested that they may be able to compete with coactivators for binding to PPAR γ 1. We tested, in a competition ELISA, whether a synthetic peptide, NBM239, could compete away the binding of the coactivator, activating signal cointegrator 2 (ASC-2), from PPAR γ 1. This was achieved by adding to wells containing immobilized hPPAR γ 1 increasing amounts of NBM239 with a fixed pfu of a T7 phage expressing the nuclear receptor AF2-interacting fragment of ASC-2 (amino acids 254–321). After incubation and washing, the amount of ASC-2 retained was detected using anti-T7 phage horseradish peroxidase (HRP)-conjugated antibody. NBM239 was able to compete away the coactivator-phage from hPPAR γ 1 in a dose-dependent manner, demonstrating that, *in vitro*, these peptides are avid competitors of coactivator binding (Fig. 6A).

Given this finding, we hypothesized that these peptides might be capable of functioning as peptide antagonists of PPAR γ 1 transcriptional activity by blocking receptor-coactivator binding in cells. Thus, transcriptional interference assays were performed by testing the ability of PPAR-interacting peptides to repress PPAR γ 1-mediated transcription of transfected DR1-luciferase (DR1-Luc) or phosphoenolpyruvate carboxykinase (–2000/+73)-luciferase (PEPCK-Luc) reporters. As a comparison, we also tested the 19-amino acid peptide PGC1 α L2, which contains the PPAR γ 1-interacting domain of the coactivator PGC-1 α , in transcriptional interference assays.

Transfection of PGC1 α L2, NBM239, and NBM242 all repressed PPAR γ 1 transcription of both DR1-Luc and PEPCK-Luc in a dose-dependent manner (Fig. 6B). The relative competitive potencies of these three peptides could be attributed to differences in affinity for PPAR γ 1 or protein expression levels. In addition to inhibiting rosiglitazone-activated transcription, NBM239 is capable of inhibiting the high constitutive activity of PPAR γ 1 by 50% on DR1-Luc (Fig. 6B, panel 3) or by 75% on PEPCK-Luc (Fig. 6B, panel 4). The specificity of inhibition was demonstrated by the fact

that basal expression of the reporter genes in the absence of cotransfected PPAR γ 1 was not affected by the peptides (data not shown). In contrast, a previously identified peptide that does not bind to PPAR γ 1, H60, has no effect on PPAR γ 1 transcription on either DR1-Luc or PEPCK-Luc (data not shown). Thus, these peptides represent novel antagonists of PPAR γ 1 that function by blocking coactivator recruitment to the receptor and inhibiting transcription of transfected reporters.

PPAR Peptides Have Higher Affinities for PPAR LBDs Than Coactivator Peptides

To determine whether these peptides exhibit the high affinity for the PPARs that would facilitate their use as peptide antagonists of endogenous genes, we performed fluorescence polarization assays to establish the affinity of selected peptides for the PPARs. We first assessed the affinity of LXXLL motifs from PGC-1 α and TRAP220 for the PPAR LBDs. The 5-carboxyfluorescein (5-FAM)-labeled peptides containing nuclear receptor interaction motifs from PGC-1 α and TRAP220 bind the PPAR LBDs with distribution constant (K_D) values ranging from 732 to 2754 nM, similar to K_D determined for the ER α LBD (Fig. 7A, *right graph* and *table*).

We then determined the affinities of the following 5-FAM-labeled peptides for PPAR α , PPAR γ , PPAR δ , and ER α LBDs in the absence of ligand: NBM131, NBM239, and NBM131mut (a control peptide consisting of the NBM131 peptide with the LXXLL mutated to AXAA). NBM131mut had no detectable affinity for any LBD tested (data not shown). NBM131 had K_D values of 87, 77, and 308 nM, and NBM239 had K_D values of 78, 78, and 48 nM for PPAR α , PPAR γ , and PPAR δ LBDs, respectively (Fig. 7A, *left* and *middle graphs*, and *table*). In contrast, the K_D values of NBM131 and NBM239 for ER α LBD were >20,000 and 8319 nM, respectively. The affinity measurements did not appreciably differ in the presence of ligands for each of the receptors (data not shown). These affinity measurements revealed that the peptides we identified have 1 order of magnitude higher affinity and selectivity for the PPARs than coactivator peptides, highlighting their potential utility as peptide antagonists of endogenous PPAR target genes.

PPAR-Selective Peptide Antagonist, NBM131, Represses Endogenous Gene Transcription in HepG2 Cells

The ability of the PPAR peptide antagonists to efficiently repress transcription on DR1-Luc and PEPCK-Luc reporter genes (Fig. 6B) and their high affinity (Fig. 7A) suggested that they might be able to function as antagonists of endogenous target genes. We screened the 540 peptides we had cloned to identify those that display a low level of binding to other receptors in mammalian two-hybrid assays. In this man-

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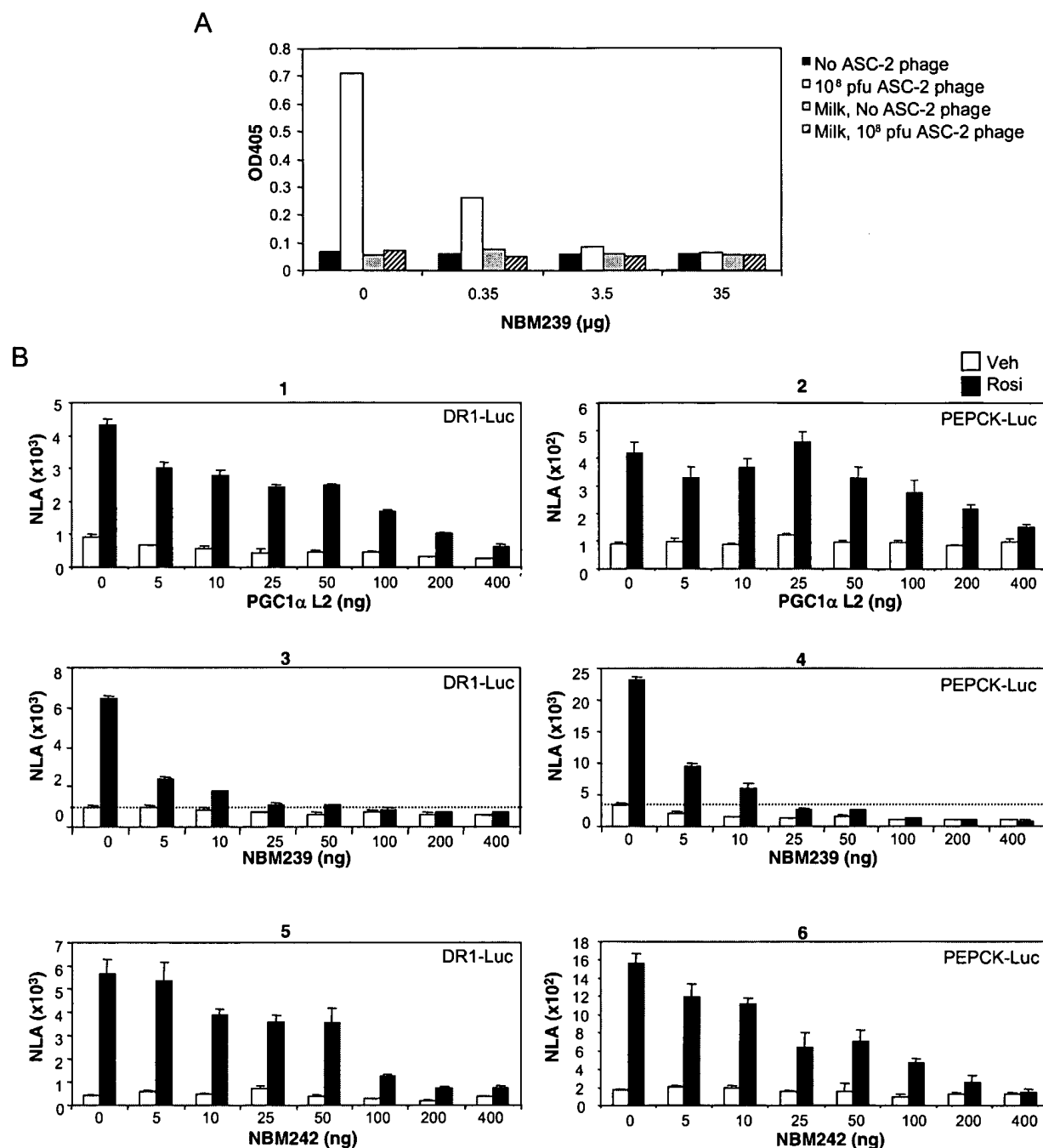


Fig. 6. PPAR Peptides Compete with Coactivators to Inhibit PPARE-Containing Reporter Gene Expression

A, Competition ELISA using synthetic NBM239 to compete away a coactivator-phage expressing the nuclear receptor-interacting domain of ASC-2 (amino acids 254–321, ASC-2 phage) from purified PPAR γ 1 *in vitro*. **B**, Effect of PGC1 α L2 (panels 1 and 2), NBM239 (panels 3 and 4), and NBM242 (panels 5 and 6) on PPAR γ 1 transcription of transfected DR1-luciferase (DR1-Luc, *left panels*) and PEPCK-luciferase (PEPCK-Luc, *right panels*) reporter genes in cis-trans assays. HeLa cells were transfected with pcDNA3.1nV5-PPAR γ 1, DR1-Luc or PEPCK-Luc, increasing amounts of the pM-peptide or pM5.1-peptide indicated on the x-axis, and CMV- β gal. Peptide amounts were balanced with empty pM or pM5.1 vector. *White bars* (□) were treated with vehicle (Veh); *black bars* (■) were treated with 1 μ M rosiglitazone (Rosi). Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells.

ner, we identified a PPAR-selective peptide, NBM131, which interacts with the PPARs (Fig. 2), but does not interact with most other nuclear receptors in the absence of exogenous ligands (supplemental Fig. 1A

published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). As expected, transfected NBM131 efficiently inhibited PPAR γ 1 transcriptional activity on

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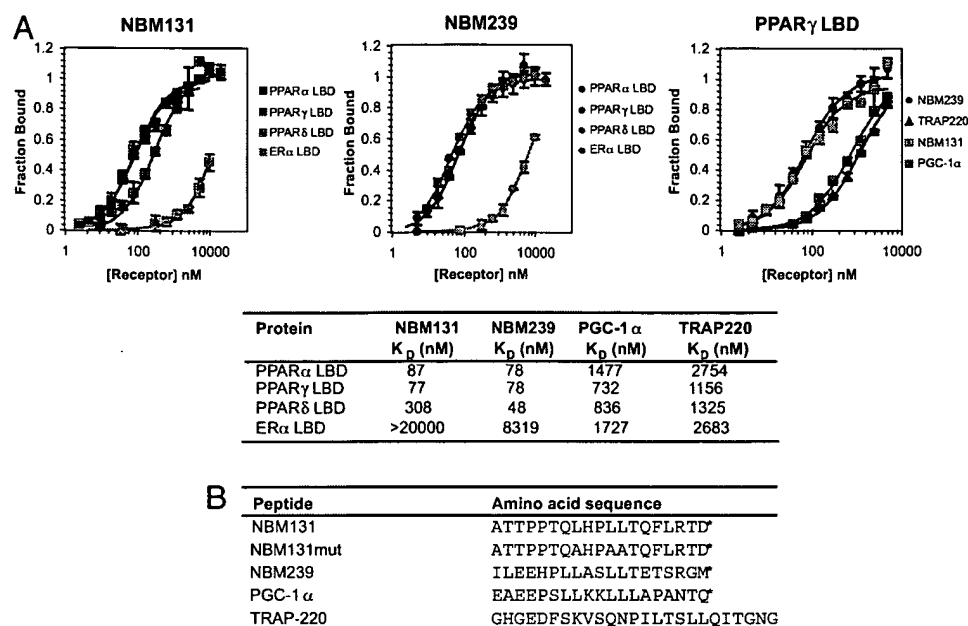


Fig. 7. PPAR Peptides Have Higher Affinities for PPAR LBDs Than Coactivator Peptides

A, Affinities (K_D) of 5-FAM-labeled NBM131 (left graph) and NBM239 (middle graph) for the LBDs of PPAR α , PPAR γ , PPAR δ , and ER α were determined by fluorescence polarization in the absence of ligand. Affinities of the nuclear receptor interaction domains of PGC-1 α and TRAP220 were also determined for each of the LBDs, and the data are presented graphically for PPAR γ LBD (right graph). The K_D for each peptide is indicated in the table below the graphs. Data presented are the average of two independent experiments. **B**, Amino acid sequences of PPAR-interacting peptides used in this experiment. *, The fluorescent peptides synthesized for use in fluorescence polarization assays had an extra cysteine at the C terminus.

both DR1-Luc and PEPCK-Luc reporter genes (Fig. 8A, top), but had no effect on the transcriptional activity of other nuclear receptors (supplemental Fig. 1, B and C).

We created a control version of this peptide, NBM131mut4, which has the LXXLL mutated to LXXAA. Mutations of the leucines within the LXXLL motif disrupted the ability of NBM131mut4 to interact with any of the PPARs (supplemental Fig. 1D). Accordingly, NBM131mut4 lacked the ability to repress PPAR γ 1 transcription of DR1-Luc and PEPCK-Luc (Fig. 8A, bottom).

We generated adenoviruses expressing NBM131 and NBM131mut4 fused to the Gal4DBD and showed that both peptides were expressed at comparable levels when HepG2 cells were infected with the adenoviruses, as illustrated by Western blot analysis of the whole cell extracts using an anti-Gal4DBD antibody (Fig. 8B). We further confirmed that the adenoviral-mediated expression of NBM131, but not NBM131mut4, potently represses transcription of a transfected DR1-Luc reporter gene, in a manner similar to the transfected peptide (supplemental Fig. 1E).

To address whether NBM131 could function as a peptide antagonist of endogenous PPAR-regulated gene expression, we used quantitative PCR to confirm the expression of several known PPAR targets in our model system, HepG2 cells. Adipose differentiation-related

protein (ADRP) has been shown to be regulated by agonists of all three PPAR subtypes *in vitro* and *in vivo*, and, as expected, we found that rosiglitazone induces the expression of ADRP (data not shown) (43). 3-Hydroxy-3-methylglutaryl coenzyme A synthase 2 (HMGCS2) is a well-characterized PPAR α target gene in the liver that produces the mitochondrial HMGCS2 enzyme, which catalyzes a key reaction in the ketogenesis pathway (44). As expected, we found that GW7647, a PPAR α agonist, induces the expression of HMGCS2 (data not shown). However, interestingly, we found that HMGCS2 can be induced to an even higher degree by rosiglitazone (data not shown).

To determine whether our PPAR-selective peptide, NBM131, could inhibit the expression of these PPAR γ 1 target genes, we infected HepG2 cells with the adenoviruses expressing either NBM131 or NBM131mut4. We found that introduction of NBM131 resulted in inhibition of the rosiglitazone-stimulated induction of both ADRP and HMGCS2, as compared with cells infected with the control adenovirus NBM131mut4 (Fig. 8C). In conclusion, we have been able to use a high-affinity 19-amino acid LXXLL-containing peptide to inhibit transcription of endogenous PPAR γ 1 target genes, further validating the approach of targeting the nuclear receptor-coactivator interaction as a means by which to modulate nuclear receptor transcriptional activity.

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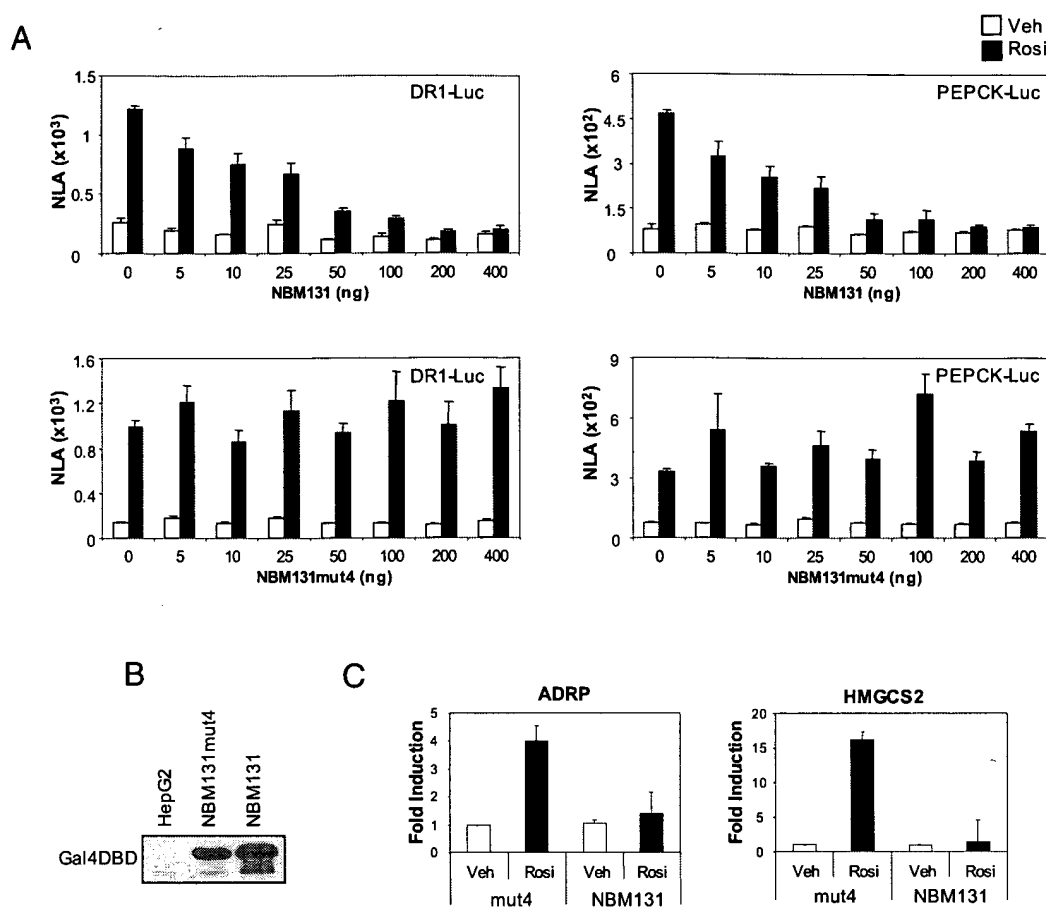


Fig. 8. PPAR-Selective Peptide Antagonist, NBM131, Represses Endogenous Gene Transcription in HepG2 Cells

A, Effect of NBM131 (*top panels*) and NBM131mut4 (*bottom panels*) on PPAR γ transcription of transfected DR1-luciferase (DR1-Luc, *left panels*) and PEPCK-luciferase (PEPCK-Luc, *right panels*) reporter genes in cis-trans assays. HeLa cells were transfected with pcDNA3.1nV5-PPAR γ 1, DR1-Luc or PEPCK-Luc, increasing amounts of the pM5.1-peptide indicated on the x-axis, and CMV- β gal. Peptide amounts were balanced with empty pM5.1 vector. *White bars* (\square) were treated with vehicle (Veh); *black bars* (\blacksquare) were treated with 1 μ M rosiglitazone (Rosi). Results are expressed as NLA (normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells. **B,** Expression of peptides in HepG2 cells infected with adenoviruses expressing NBM131 or NBM131mut4 by Western blot analysis using an anti-Gal4DBD primary antibody and an antirabbit IgG HRP-conjugated secondary antibody. **C,** Effect of NBM131 and NBM131mut4 on endogenous target gene expression of adipocyte differentiation-related protein (ADRP) and 3-hydroxy-3-methylglutaryl coenzyme A synthase 2 (HMGCS2) by real-time RT-PCR. *White bars* (\square) were treated with vehicle (Veh); *black bars* (\blacksquare) were treated with 1 μ M rosiglitazone (Rosi). Sequences of primers of genes used are indicated in supplemental Fig. 1F. Results are normalized to the NBM131mut4-infected, vehicle-treated sample.

DISCUSSION

Nuclear receptor-mediated target gene expression is a multistep process that involves ligand binding, association with target gene promoters, coactivator recruitment, and assembly of the transcriptional machinery. Although each step in this process can be targeted for the development of new drugs, most of the current drugs target only the initial step. In the past, we have tried to target coactivator recruitment, and to this end, we have identified selective peptides for a number of nuclear receptors and used them to inhibit nuclear receptor-mediated activation of transfected reporter genes in cells. However, we have not been able to use this approach to inhibit endogenous target gene ex-

pression. This difficulty is primarily thought to be due to the low affinity of peptides for their respective receptors. In the current study, we have successfully identified peptides with nanomolar affinity for the PPARs, and most importantly, we have used these peptides to inhibit transcription of endogenous PPAR target genes. Notably, one of our peptide antagonists, NBM131, inhibited the rosiglitazone-induced transcription of ADRP and HMGCS2.

Although our studies were focused on PPAR, it is clear that the general approach of blocking nuclear receptor-coactivator interactions can be applied to target other receptors and biologies. Aromatic β -amino ketones have been identified that covalently bind to thyroid receptor (TR) in a pocket distinct from the

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agonist-binding pocket, and these compounds inhibit TR-coregulator interaction *in vitro* and TR transcriptional activity on a transfected reporter (45). Additionally, ketoconazole, a commonly used antifungal drug, inhibits the interaction between pregnane X receptor (PXR) and SRC-1 *in vitro* and *in vivo*, leading to an inhibition of PXR-mediated drug metabolism *in vivo* (46). These compounds each bind to the receptor at sites distinct from the agonist-binding pocket and inhibit the interaction between nuclear receptors and coactivators, thereby blocking receptor-mediated transcription. These studies support our hypothesis that the nuclear receptor-coactivator interaction can be targeted to modulate receptor-mediated transcription. Furthermore, they demonstrate the applicability of this approach to nuclear receptors other than the PPARs.

This approach may be of clinical value in the setting of hormone-dependent cancers. For example, the mainstay of therapy in breast and prostate cancers is anti-hormone therapy; however, the progression of initially anti-hormone-responsive cancers to an anti-hormone-refractory state is common, necessitating the development of drugs that can block the transcriptional activity of nuclear receptors via mechanisms other than classic antagonism. The onset of resistance to anti-hormone therapy highlights the unmet need for mechanistically distinct antagonists. The significance of the nuclear receptor-coactivator interaction for nuclear receptor-mediated activation of gene transcription suggests that targeting this interface offers a novel and complementary mechanism by which to inhibit a receptor's transcriptional activity. This approach may be used for the treatment of refractory tumors or disease processes in which a competitive antagonist of the receptor is difficult to develop.

The results of this study should serve as a prototype for the rational development of peptidomimetics as a mechanism by which to modulate nuclear receptor activity. Small molecule peptidomimetics obviate some of the disadvantages of using peptides as drugs, e.g. low bioavailability, poor transport into the bloodstream, and rapid excretion (47). By defining the local and global conformational parameters of a biologically active peptide and optimizing the constituents of the backbone amino acids, a peptidomimetic can be developed that retains the peptide's effect with improved stability, bioavailability, and transport across cell membranes (47, 48). Techniques such as polyarginine modification or the addition of other cell-penetrating peptides can facilitate transport (49, 50). However, each of these approaches limits oral bioavailability and increases the costs of pharmaceutical production. Limiting the peptide length is also an important consideration in designing peptidomimetics with increased stability and transport. We have demonstrated that we are able to narrow down a 19-amino acid peptide to eight amino acids and still retain binding to PPAR γ 1 in the absence and presence of ligand. Although this eight-amino acid peptide was not capa-

ble of inhibiting PPAR γ 1 transcription (data not shown), we believe that with further optimization, it may be possible to develop peptidomimetics targeting this interaction.

It has been previously demonstrated that the dipeptide G3335 (H-Trp-Glu-OH) is a PPAR γ antagonist that blocks the ability of rosiglitazone to stimulate an interaction between the PPAR γ and RXR α LBDs (51). However, the specificity, mechanism, and effect on endogenous target genes of G3335 have not been characterized. In assays measuring the PPAR γ LBD-CREB binding protein interaction and repression of reporter gene expression, the IC₅₀ of G3335 was 8.67 μ M and 31.9 μ M, respectively. In contrast, our peptide antagonists possess nanomolar affinity for the PPAR LBDs and a very high degree of specificity for the PPARs over other nuclear receptors, both of which are requisite for their use *in vivo*.

Interestingly, 98% of the PPAR-interacting peptides that we identified also interact directly with activated RXR α bound to 9cisRA. We have previously shown the same to be the case for LRH-1-interacting peptides (29). Although most of our PPAR peptides interact with RXR α in a ligand-dependent manner, the PPAR-selective peptide NBM131 does not inhibit transcription of RXR α or of any of the other receptors that heterodimerize with RXR α . These data suggest that the peptides may interact with another surface on RXR α that is exposed upon ligand binding, or that they lack the high affinity for RXR α needed to compete with coactivators for binding.

We have demonstrated that PPAR γ 1 prefers peptides that have a very strong consensus binding motif consisting of HPLLXXLL. Although it has been demonstrated previously that residues C-terminal to the LXXLL motif play important roles in binding to PPAR γ 1 and other nuclear receptors, no consensus emerged in the amino acids C-terminal to the LXXLL within the peptides that we identified (21). The amino acids in the –1 and –2 positions with respect to the LXXLL motif have been shown to stabilize the nuclear receptor-coactivator interaction through various noncovalent interactions, including hydrogen bonding, van der Waals forces, and salt bridge formation (25). From peptide phage display screens performed with other nuclear receptors, it has become clear that residues in the –1 and –2 positions correlate with receptor-binding specificity (23–29, 52). However, no consensus correlating the nature of the –3 amino acid with receptor-binding specificity has been previously established.

Our molecular modeling predicts that the –3 histidine found within our HPLLXXLL motif interacts with two residues within PPAR γ 1: K319 and E471. E471 forms one end of the charge clamp, and mutation of this residue abrogates interaction of our peptides with PPAR γ 1. Furthermore, our data predict that the binding of the peptides to PPAR γ 1 is stabilized by a salt bridge between the –3 histidine of the peptide and K319 of PPAR γ 1. K319 in helix 4 is important for the

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formation of an intramolecular bond with D475 in helix 12 of PPAR γ 1, and this may contribute to stabilization of helix 12 and coactivator binding (53). Indeed, K319 may form a hydrogen bond with H687 that is found at the –3 position with respect to the second LXXLL motif of SRC-1 (amino acids 690–694), which may explain why the K319A mutant has lower constitutive activity (53, 54). We found that the K319A mutation significantly reduces the interaction between apo- and ligand-activated PPAR γ 1 and NBM242, suggesting that K319 may be important for stabilizing interactions of the receptor with coactivators in both the absence and presence of ligand. In addition, K319 is a conserved residue in PPAR α , PPAR γ , and PPAR δ , a fact that may explain why the peptides we isolated bind to all three PPAR subtypes.

The high level of constitutive activity is an interesting aspect of PPAR γ 1 biology. The lack of available antagonists that can inhibit this constitutive activity has hampered our knowledge of the full array of biological effects of this receptor. The development of peptide antagonists that can repress the constitutive activity of PPAR γ 1 will advance our knowledge of the biological roles played by this receptor. This will ultimately be important in understanding the therapeutic applications and side effects of PPAR γ ligands used clinically.

The etiology of the constitutive activity of PPAR γ 1 is not altogether clear. Because an endogenous ligand for PPAR γ 1 has yet to be confirmed, one hypothesis is that in cells, PPAR γ 1 is bound to a low-affinity fatty acid or other ligand, which causes the recruitment of coactivators responsible for the constitutive activity of PPAR γ 1 (53). Agonist binding increases the stability of the AF-2 structure, further strengthening the recruitment of coactivators to enhance transcription. It stands to reason that a coactivator with high affinity for the unliganded receptor could increase the constitutive activity of the receptor, obviating the need for any ligand; discovery of a PPAR-binding motif may help identify novel coactivators that can function in this regard. Interestingly, mutation –6 of NBM242 has the ability to bind with high ligand independence. This raises the possibility that the constitutive activity of PPAR γ 1 could be explained by coactivator recruitment to a conformation of PPAR γ 1 induced either in the absence of ligand or in the presence of an unconfirmed endogenous ligand. It is worth noting that using NBM239, we are able to partially inhibit the constitutive activity of PPAR γ 1, demonstrating that the constitutive activity can be partially blocked through the use of LXXLL-containing peptide antagonists of PPAR γ 1.

It has been suggested that helix 12 of constitutively active nuclear receptors contains amino acids that are required for their high basal activity (53). A heterozygous dominant-negative mutation within helix 12 of PPAR γ 1, P467L, has previously been associated with early-onset type 2 diabetes mellitus and hypertension (55). This mutation perturbs the orientation and dynamic properties of helix 12, likely resulting in abro-

gated coactivator recruitment and reduced constitutive PPAR γ 1 transcriptional activity. Of note, P467 is found within a sequence motif consisting of HPLLQEIY, a conserved motif found in helix 12 of all three PPAR subtypes. Although this motif is not identical to the motif we identified, it is quite similar, especially considering the flexibility in the +4 and +5 positions that we observed. The presence of this motif is interesting especially in light of the finding that the extended helix 12 of one PPAR γ molecule in an inactive conformation contacts the charge clamp of another apo-PPAR γ molecule in a crystallographically related homodimer that is in an active conformation (54). This finding raises the question of whether this motif is important for the interaction. Whether such an intermolecular interaction involving this motif is relevant to the PPAR/RXR heterodimer is not known.

The results of this study support the hypothesis that targeting the nuclear receptor-coactivator interaction surface could be a viable mechanism of inhibiting nuclear receptor function *in vivo*. In addition to elucidating some of the molecular determinants of PPAR-coactivator interactions, this work lends support for the rational design of peptidomimetics that can be used to target this interaction in the broad range of physiological and pathological processes in which nuclear receptors are implicated.

MATERIALS AND METHODS

Biochemicals

Rosiglitazone, carbaprostacyclin, and 22(R)-OH-cholesterol were purchased from Cayman Chemical (Ann Arbor, MI). Clofibrate, GW7647, 9 α cisRA, 17 β -estradiol, dexamethasone, chenodeoxycholic acid, rifampicin, T $_3$, and all-trans retinoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Methyltrienolone (R1881) and promegestone (R5020) were purchased from PerkinElmer, Inc. (Waltham, MA). 1,25-Dihydroxy-vitamin D $_3$ was purchased from BIOMOL International, L.P. (Plymouth Meeting, PA). GW7845 was a gift from Glaxo-SmithKline (Research Triangle Park, NC). Mutagenesis and quantitative PCR oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA).

Plasmids

Cytomegalovirus (CMV)- β gal was purchased from CLONTECH Laboratories, Inc. (Mountain View, CA). pBSII was purchased from Stratagene (La Jolla, CA). Mammalian expression constructs for nuclear receptors were obtained as described previously (56). cDNAs for PPARs were obtained: PPAR γ 1 (B. Spiegelman), PPAR δ (GlaxoSmithKline), and PPAR α (HepG2 cells). Mammalian expression vectors pcDNA3.1nV5-PPAR γ 1, VP16-GwA, VP16-GwA-PPAR γ 1, VP16-GwA-PPAR γ 2, VP16-GwA-PPAR δ , and VP16-GwA-PPAR α were constructed as described previously (58). The nuclear receptor reporter genes used were: 3X-ERE-tata-Luc (for ER α , ER β , ERR α , ERR β , and ERR γ) (57), DR1-Luc (for PPAR γ 1, RXR α , and hepatocyte nuclear factor-4 α 2) (R. Heyman), mouse mammary tumor virus-Luc (for AR and progesterone receptor), SHP-1400-Luc (for LHRH-1) (S. Klierer), DR5-tk-Luc (for RAR α) and LXRE-tk-Luc (for liver X receptor α) (D. Mangelsdorf), and 5XGal4TataLuc3 (25). The PEPCK

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(–2000/+73)-luciferase reporter gene was a gift from Dr. Edwards A. Park (University of Tennessee Health Science Center, Memphis, TN). pM-PGC1 α L2 was constructed as described previously (30). Peptides were cloned into pM5.1, a vector derived from pM (CLONTECH) that enables the peptides to be cloned as N-terminal fusions to the Gal4DBD. The mutations of NBM242 and the K319A and E471A mutations of PPAR γ 1 were generated by QuikChange Site-Directed Mutagenesis Kit (Stratagene) using primers with the desired mutations. All PCR products were sequenced to ensure the fidelity of the resulting constructs.

Production and Purification of Full-Length Recombinant hPPAR γ 1 and hRXR α and DNA-Binding Assay

hPPAR γ 1 and hRXR α were purified as previously described for the purification of other nuclear receptors (37). After expression of the biotinylated proteins in Sf9 insect cells, hPPAR γ 1 and hRXR α were purified by batch method using monomeric avidin resin (Promega Corp., Madison, WI) or using fast performance liquid chromatography and Streptavidin Mutein Matrix resin (Roche, Indianapolis, IN), as has been previously described. The proteins were run on reducing SDS-PAGE gels and detected by Coomassie blue staining (37). To test the functionality of the purified proteins, a DNA-binding assay was performed. A 96-well plate was prepared as follows: well coated with 10 μ g neutravidin (Thermo Fisher Scientific, Inc., Pierce Biotechnology, Rockford, IL) in 100 mM NaHCO $_3$ (pH 8.5) for 2 h at room temperature, blocked for 1 h with 2% milk in NaHCO $_3$, washed five times with PBST (PBS + 0.1% Tween 20), incubated with 2 pmol of double-stranded 5'-biotinylated oligonucleotides (diluted in PBST) containing the aP2 PPRE for 1 h (5Bio-GATCTAAAC-TAGGTCAAAGGTCATGCG annealed with 5Bio-GATCCG-CATGACCTTTGACCTATT), blocked with 5 mM biotin for 1 h, and washed five times with PBST. Equal molar quantities of purified biotinylated hPPAR γ 1 and/or purified biotinylated hRXR α (1 μ g each) were allowed to bind to the PPRE overnight at 4 C in PBST in the presence of ligand. Ligands used were 1 μ M of the nonthiazolidinedione tyrosine-based PPAR γ ligand GW7845 or 1 μ M 9cisRA. The wells were washed five times with PBST to remove unbound proteins. Proteins were eluted by boiling in Laemmli 4X buffer (Bio-Rad Laboratories, Inc., Hercules, CA), run on a reducing SDS-PAGE gel, and detected by Coomassie blue staining.

Affinity Selection of PPAR Binding Sequences

M13 phage particles displaying PPAR-selective peptides were obtained as described previously (25, 30, 52). In brief, 2 μ g of purified baculovirus-expressed, biotinylated, purified hPPAR γ 1 was diluted in 100 μ l of 100 mM NaHCO $_3$ (pH 8.5) and either 1 μ M rosiglitazone solubilized in 100% ethanol or the equivalent volume of ethanol. The protein was allowed to bind the plastic wells overnight at 4 C. Alternatively, to prepare wells for screening the DNA-bound hPPAR γ 1/hRXR α heterodimer, wells of a 96-well plate were prepared as described above for the DNA-binding assay. The wells were treated with approximately 10 10 phage particles of a phage peptide library preblocked in 100 μ l of MPBS (2% nonfat dry milk in PBS) for 1 h, and panning was done as described previously (30). Phage eluted from the target were amplified in *Escherichia coli* DH5 α F' cells (Invitrogen, Carlsbad, CA) for 5 h at 37 C, and the supernatant containing amplified phage was collected for use in subsequent rounds of panning for a total of three rounds of panning. Enrichment of PPAR-binding phage was confirmed by a phage ELISA as described previously (25). PCR amplification of the peptides was performed using 0.4 μ l of the amplified phage from bacterial supernatants that showed significant enrichment of target-binding phage. The PCR products were purified and digested with *Xba*I and *Xho*I before ligation into pM5.1 for mammalian

two-hybrid analysis. The peptide sequences were deduced by DNA sequencing. Construction of the LXXLL M13 phage library was described previously (25), and the SS-X $_6$ -S M13 phage library was a gift from D. J. Kenan (Duke University Medical Center, Durham, NC).

Coactivator-Phage Competition ELISA

A T7 phage expressing the nuclear receptor-interacting fragment of ASC-2 (amino acids 254–321) was obtained from a screen of a T7 library performed in our laboratory (Dwyer, M. A., and D. P. McDonnell, unpublished data). The competition ELISA was performed by immobilizing hPPAR γ 1 directly on the plastic well overnight at 4 C as described above for the affinity selection of PPAR binding sequences. This was followed by blocking and incubating with a combination of 10 8 pfu of ASC-2 coactivator-phage plus the synthetic NBM239 peptide (see below for details on synthesis) for 3 h. The wells were washed 10 times with PBST, and the bound phage were detected by ELISA, using anti-T7 phage HRP-conjugated antibody (Novagen, San Diego, CA). Bound antibody-enzyme conjugate was detected by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich) in the presence of 0.05% H $_2$ O $_2$ for 15 min, and absorbance was measured at 405 nm in a Multiskan MS microplate reader (Labsystems, Inc., Helsinki, Finland).

Mammalian Cell Culture and Transient Transfection Assays

HepG2 human hepatoma cells and HeLa human cervical carcinoma cells were obtained from American Type Culture Collection (Manassas, VA), maintained in MEM (Invitrogen) supplemented with 8% fetal bovine serum (HyClone Laboratories, Logan, UT), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) in a humidified 37 C incubator with 5% CO $_2$. All mammalian two-hybrid analyses were performed in HepG2 cells. Cis-trans assays were performed in HeLa cells. HepG2 cells were seeded in wells of 96- or 24-well plates coated with 0.1% gelatin for 10 min at 25 C. For transient transfections, cells were split in 24- or 96-well plates 24 h before transfection. On the day of transfection, the medium was aspirated and cells were washed once with PBS. Cells were transfected with 3 μ g of total plasmid per triplicate sample in 24-well or 600 ng in 96-well plate format, using Lipofectin according to manufacturer's protocol (Invitrogen). DNA-Lipofectin mix was added and incubated for 5 h (HepG2) or 4 h (HeLa). The transfection was stopped by aspirating the DNA-Lipofectin mix and replacing with phenol red-free MEM (Invitrogen) supplemented with 8% charcoal-stripped serum (HyClone), nonessential amino acids, and sodium pyruvate (Invitrogen). All ligand stocks were dissolved in ethanol or DMSO before use in cell culture, and cells were treated with ligands added to phenol red-free MEM. A saturating concentration of ligand was added to the cells either immediately or 24 h post-transfection: 1 μ M rosiglitazone (for PPAR γ), 10 μ M carbaprostacyclin (for PPAR δ), 100 μ M clofibrate (for PPAR α), 100 nM GW7647 (for PPAR α), 1 μ M 9cisRA (for RXR α), 100 nM 17 β -estradiol (for ER α and ER β), 100 nM all-trans retinoic acid (for RAR α), 10 nM R1881 (for AR), 100 nM dexamethasone (for glucocorticoid receptor), 100 nM R5020, 100 nM T3, 100 nM 1,25-dihydroxy-vitamin D $_3$, 50 μ M chenodeoxycholic acid (for farnesoid X receptor), 10 μ M 22(R)-OH-cholesterol (for LXR), and 10 μ M rifampicin (for PXR). Cells were lysed 40–48 h after transfection and assayed for luciferase and β -galactosidase activities using a Fusion Universal Microplate Analyzer (PerkinElmer). Results are expressed as normalized luciferase activity (NLA, normalized with β gal for transfection efficiency) \pm SEM per triplicate sample of cells. Results presented were typical of three independent transfection experiments, each performed with triplicates of each experimental condition.

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Molecular Modeling Studies

Structural models of PPAR γ LBD in complex with the NBM242 peptide were generated using Pymol molecular graphics and modeling package (41) and the crystal structure coordinates of the LBD of PPAR γ in complex with the SRC-1 peptide (PDB: 1FM6) (42). The residues of the SRC-1 peptide were mutated *in silico* to the residues of the NBM242 peptide, and the most frequently occurring amino acid rotamer was selected.

Western Blot Analysis

HepG2 cells were transfected or infected as indicated, and after 48 h, cells were lysed in X-400 buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 400 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and Protease Inhibitor Cocktail 3 (Calbiochem, San Diego, CA). Laemmli 4X buffer (Bio-Rad) was added to the samples after Bradford protein estimation (Bio-Rad). The samples were boiled for 10 min at 95 C, loaded on a reducing SDS-PAGE gel, and transferred to nitrocellulose membrane (Amersham, Buckinghamshire, UK). Expression of peptides was detected with the anti-Gal4DBD antibody sc577 diluted 1:500 in MPBST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and antirabbit IgG HRP-conjugated secondary antibody NA934 diluted 1:4000 in MPBST (Amersham). Expression of PPAR γ 1, PPAR γ 1K319A, and PPAR γ 1E471A was detected with the anti-PPAR γ antibody E-8 diluted 1:1000 in MPBST (Santa Cruz Biotechnology, Inc.) and antimouse IgG HRP-conjugated antibody NA931 diluted 1:4000 in MPBST (Amersham).

Preparation of Synthetic Peptides

Peptides were synthesized by standard Fmoc protocol on PAL-PEG-PS resin (Applied Biosystems, Foster City, CA) utilizing a 396 MPS automated peptide synthesizer (Advanced ChemTech, Louisville, KY). All peptides were cleaved from the resin with 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane, yielding an amide on the peptide's C terminus. Purification was done by reverse-phase HPLC with a Vydac C18 column and a gradient elution by solvents A (99% H₂O, 1% CH₃CN, and 0.1% trifluoroacetic acid) and B (95% CH₃CN, 5% H₂O, and 0.1% trifluoroacetic acid). Molecular mass and purity were confirmed via LCMS-ESI. Peptides were then lyophilized and stored at –80 C.

Fluorescence Polarization

5-FAM-labeled fluorescent peptides were ordered with a cysteine at the C terminus (American Peptide Co., Inc., Sunnyvale, CA). Mass spectral analysis by electrospray and HPLC analysis revealed peptides of correct molecular weight with purities between 95.3% and 97.5%. The peptides were solubilized in water to a concentration of 1 mg/ml. The buffer used to dilute the protein and peptides contained 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20. Fluorescence polarization was measured using the PerkinElmer α -HT FP machine (PerkinElmer) with excitation at 485 nm and emission at 535 nm for fluoresceinated peptides. Data presented are the average of two independent experiments.

Adenoviral Construction and Infection

Adenoviruses expressing NBM131 or NBM131mut4 were generated using the ViraPower Adenoviral Expression System according to manufacturer's protocol (Invitrogen). In brief, viruses were made by subcloning the peptide-Gal4DBD fusions into pENTR-D-TOPO (Invitrogen), followed by recom-

ination into pAd/CMV/V5-DEST (Invitrogen). The PacI-digested pAd/CMV/V5-DEST constructs containing either NBM131 or NBM131mut4 were transfected using FuGENE 6 (Roche) in 293A cells (Invitrogen), and adenoviruses were produced. Recombinant adenoviruses were purified by CsCl density gradient centrifugation. The purified virus stocks were desalted over Sephadex G-25 columns (Amersham) and eluted in sterile PBS. Glycerol was added for a final concentration of 10%, and the virus stocks were aliquoted and stored at –80 C until used. Infectious viral titers were calculated using the end-point dilution assay. HepG2 cells were infected with adenoviruses by diluting the purified adenovirus to MOI 20 in a small volume of media and infecting cells on a rocker at room temperature for 2 h. The infection was stopped by aspirating the virus-containing media and replacing with fresh growth media. Twenty-four hours after infection, cells were treated with ligand for 24 h, and RNA was harvested for quantitative PCR analysis.

RNA Isolation and Quantitative Real-time PCR

For RNA isolation, HepG2 cells were seeded in six-well plates in MEM as described above. Cells were infected 24 h after plating. Twenty-four hours after infection, cells were treated with 1 μ M rosiglitazone or 100 nM GW7647 for an additional 24 h. Cells were then harvested and total RNA of HepG2 cells was isolated using Aurum Total RNA Kit according to manufacturer's protocol (Bio-Rad). Total RNA (1 μ g) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed using the Bio-Rad iCycler Realtime PCR System (Bio-Rad) using 0.1 μ l cDNA, 10 μ M each primer, and iQ SYBR Green Supermix detection reagent (Bio-Rad). Data are the mean \pm SEM of triplicate samples. A control sample without reverse transcriptase was performed to exclude genomic DNA contamination. The melt curves for each primer were also obtained to confirm that a single amplicon was obtained. The expression data were normalized to the endogenous control acidic ribosomal phosphoprotein P0 (36B4). The expression of 36B4 was not affected by adenoviral infection or by treatments. The primers were designed using GenScript or IDT Primer Quest Tool. The sequences of the primers are listed in supplemental Fig. 1F.

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